Experimental Study of Bio-Hydrogen Production by \textit{Clostridium beijerinckii} from Different Substrates

Venko Beschkov *, Tsvetomila Parvanova-Mancheva and Evgenia Vasileva

Laboratory of Bioengineering, Institute of Chemical Engineering, Bulgarian Academy of Sciences, 1113 Sofia, Bulgaria
* Correspondence: vbeschkov@iche.bas.bg; Tel.: +359-898-447-721

Abstract: Glucose, alcohol stillage and glycerol were used as substrates for bio-hydrogen production by the newly isolated strain \textit{Clostridium beijerinckii} 6A1 under batch conditions. High molar yields of hydrogen from the studied organic substrates were observed. There was a neat difference in the metabolic pathways of substrate digestion when hexose-based substrate or glycerol were used. The products of glycerol digestion showed that a pathway with no formic acid formation as intermediate was probable. In this case, considerable concentrations of acetic and propionic acid (up to 6 g dm$^{-3}$) and small amounts of butanol were observed after 48 h. When glucose or hexose-based substrates were used, considerable amounts of formic acid (up to 6 g dm$^{-3}$), i.e., the pathway proposed for \textit{Clostridia} mixed cultures, were appropriate for the observed process of hydrogen release. For these substrates, considerable amounts of propionic acid in concentrations up to 1 g dm$^{-3}$ were observed. That is why the pathway proposed for mixed cultures seemed more appropriate for our experiments carried out with hexose-based substrates. When hexoses were used, substrate digestion stopped the formation of acetic acid, propionic acid and ethanol. Probably, these intermediates are inhibitors to the further digestion to other products.

Keywords: bio-hydrogen; dark fermentation; \textit{Clostridium beijerinckii}; hexoses; glycerol

1. Introduction

Hydrogen has been always attractive as an alternative to fossil fuels such as oil and natural gas. The main reason for such an interest is the higher density of energy per unit mass compared to other energy sources. On the other hand, there is no waste as a result of its combustion; i.e., water is produced, being feedstock for further hydrogen production [1].

A hindrance to the broad application of hydrogen as a fuel is the significant amount of energy needed for its traditional production, i.e., water splitting by electrolysis. However, thermodynamic calculations show that the energy needed for electrolysis (~4.8 kWh/Nm$^3$ \text{H}_2) is bigger than the energy of the produced hydrogen (3.55 kWh/Nm$^3$ \text{H}_2). That is why cheaper and more abundant sources of energy are required for water splitting by electrolysis. In recent years, typical energy sources for this purpose have been solar energy, wind energy and hydropower [2].

There is one way to produce hydrogen with low energy input. It is hydrogen production by microbial processes [3–10].

The feasibility of bio-hydrogen production is demonstrated by the data on electricity input for hydrogen production by different methods [7] in Table 1. The energy used for fermentation processes (1.52 kWh/Nm$^3$) is less than the energy of the produced hydrogen.

The benefits of bio-hydrogen production and utilization are multiple. First, it is an energetically beneficent process, compared to traditional electrolysis. Next, bio-hydrogen production relies on waste organic products, thus combining energy production with waste treatment. Bio-hydrogen possesses a greater energy efficiency as it can release more energy than other fuels on an efficiency basis. Bio-hydrogen is a much cleaner and more efficient
fuel because it does not emit carbon dioxide and yields water as waste and liberates energy. It is a renewable source of energy because the production process consumes various organic waste of natural origin, such as food residues, alcohol stillage, lignocellulose, etc. The lack of involvement of fossil fuels in the production of bio-hydrogen makes it a renewable and promising energy option. Bio-hydrogen use is not only restricted to traditional transport activities, but it can also be applied as feedstock in the chemical industry [11].

Table 1. Energy input for production per unit amount of hydrogen. An excerpt of Table 4 in [7]. Data for traditional electrolysis are taken from [8]. Adapted with permission from Ref. [7]. Copyright Elsevier, 2008.

<table>
<thead>
<tr>
<th>Method of Production</th>
<th>Non-Renewable Energy Use per kg Hydrogen, MJ kg⁻¹</th>
<th>Non-Renewable Energy (kWh) Use per 1 Nm³ Hydrogen</th>
</tr>
</thead>
<tbody>
<tr>
<td>Steam methane reforming</td>
<td>188</td>
<td>4.66</td>
</tr>
<tr>
<td>Dark fermentation</td>
<td>61.7</td>
<td>1.52</td>
</tr>
<tr>
<td>Photo-fermentation</td>
<td>40.1</td>
<td>0.99</td>
</tr>
<tr>
<td>Two-stage fermentation</td>
<td>39.3</td>
<td>0.97</td>
</tr>
<tr>
<td>Traditional electrolysis</td>
<td>181 ÷ 222</td>
<td>4.5 ÷ 5.5</td>
</tr>
<tr>
<td>Biocatalyzed electrolysis</td>
<td>64.8</td>
<td>1.61</td>
</tr>
</tbody>
</table>

The practical production of bio-hydrogen on a large scale presumes the use of low-cost substrates such as residues from the food industry, beverage and ethanol production, residues from agriculture, etc. Various substrates for fermentative hydrogen production have been tested. Studies with glucose, cellulose and other sugars [4–6,9,10] are available. The net stoichiometric equations for the production of hydrogen from sugars, e.g., glucose, are accompanied by the formation of low fatty acids, namely [12]:

\[
\text{C}_6\text{H}_{12}\text{O}_6 + 2\text{H}_2\text{O} = 2\text{CH}_3\text{COOH} + 2\text{CO}_2 + 4\text{H}_2, \Delta G^0 = -206.3 \text{ kJ} \quad (1)
\]

\[
\text{C}_6\text{H}_{12}\text{O}_6 = 2\text{CH}_3(\text{CH}_2)_2\text{COOH} + 2\text{CO}_2 + 2\text{H}_2, \Delta G^0 = -254.8 \text{ kJ} \quad (2)
\]

Note that the latter two reactions are exergonic, i.e., the production of bio-hydrogen becomes more energetically attractive compared to electrolysis. Additionally, there are data about the potential of glycerol as a substrate for bio-hydrogen production [12–16]. The utilization of waste glycerol from biodiesel production as a substrate for hydrogen production seems attractive [16]. The net reactions for hydrogen production from glycerol are [13]:

\[
\text{C}_3\text{H}_5\text{O}_3 + \text{H}_2\text{O} = \text{CH}_3\text{COOH} + \text{CO}_2 + 3\text{H}_2, \Delta G^0 = -70.3 \text{ kJ/mole} \quad (3)
\]

\[
2\text{C}_3\text{H}_8\text{O}_3 = \text{CH}_3\text{CH}_3\text{CH}_2\text{COOH} + 2\text{CO}_2 + 4\text{H}_2, \Delta G^0 = -254.8 \text{ kJ/mole} \quad (4)
\]

\[
2\text{C}_3\text{H}_8\text{O}_3 = \text{C}_4\text{H}_9\text{OH} + 2\text{CO}_2 + \text{H}_2\text{O} + 2\text{H}_2 \quad (5)
\]

\[
\text{C}_3\text{H}_8\text{O}_3 = \text{C}_2\text{H}_5\text{OH} + \text{CO}_2 + \text{H}_2 \quad (6)
\]

There are two main groups of microbial processes leading to hydrogen production depending on the light involvement. First, there is photo fermentation using light to produce sugars by photosynthesis and then to convert organic molecules into hydrogen and carbon dioxide. Next, there is dark fermentation when anaerobic bacteria and algae convert complex substrate molecules into hydrogen and carbon dioxide [16]. In these cases, some of the intermediates or by-products are inhibitors to bio-hydrogen production [14,17,18]. That is why there are some efforts to combine these two types of fermentation to degrade low
molecular inhibitors by light fermentation or to enhance dark fermentation by additivities, such as some metal ions and oxides, amino acids, etc. [19].

Different strains are capable of producing hydrogen: *Clostridium*, *Enterobacter*, *Escherichia coli*, *Klebsiella*, *Halanaerobium*, etc. [20–28]. The best studied varieties of bacteria belong to the genus *Clostridium*. [6,22–27]. The strain *Clostridium beijerinckii* has an important impact as a hydrogen producer in the literature [24–27].

Depending on the used strain, different metabolic pathways for hydrogen production are described. One, based on the use of strains of the genus *Clostridium*, is shown in Figure 1 [23]. It is suitable to explain the conversion of hexoses, pentoses and glycerol to hydrogen and other metabolites, including volatile fatty acids.

![Figure 1. Hydrogen production metabolic pathways in genus *Clostridium*, proposed in [23]. Hydrogen production processes are marked in blue. Reprinted with permission from Ref. [23]. Copyright Elsevier, 2021.](image)

According to this pathway, hydrogen is produced by the reduction of protons by pyruvate by Fd$_{\text{red}}$ by [FeFe] hydrogenase and from NADH by the same enzyme [23]. The resulting Acetyl-CoA is converted into ethanol and/or acetate in competitive routes. Another competitive conversion of Acetyl-CoA leads to hydrogen production, resulting finally in butanol and/or butyrate.

There also other metabolic pathways that differ in the intermediate and final fermentation products [6,21]. They are shown in Figures 2 and 3.

Figure 3 proposes different pathways for hydrogen production. However, in all cases the key intermediate is pyruvate.

Figure 2 shows the metabolic network leading to hydrogen production by *Clostridia* mixed cultures at dark fermentation [28]. It is based on glucose as the initial substrate and implies the production of fatty acids, ethanol, propanol and butanol. The hydrogen production is also considered by pyruvate reduction, whereas the produced hydrogen is consumed for the reduction of acetic acid and carbon dioxide to give methane as a competitive product. It is important to note that according to this pathway formic acid is also produced directly from pyruvate.
Figure 2. Biochemical network for hydrogen production in mixed culture [28]. Reprinted with permission from Ref. [28]. Copyright Elsevier, 2013.

On the other hand, methane formation is competitive and an undesired process during dark anaerobic fermentation, cf. Figure 2. It takes place in the presence of methanogens, following the net reaction of hydrogen consumption:

$$\text{CO}_2 + 4\text{H}_2 = \text{CH}_4 + 2\text{H}_2\text{O}$$ (7)

or by the decarboxylation of acetic acid:

$$\text{CH}_3\text{COOH} = \text{CH}_4 + \text{CO}_2$$ (8)

These two pathways, shown in Figures 2 and 3, include the formation of formic acid, which is not considered in the pathway when hexoses, pentoses and glycerol are used as substrates by *Clostridia* (Figure 1). Figure 3 also illustrates the possibility of producing
hydrogen via formate depletion as well as acetate plus lactate conversion to hydrogen and carbon dioxide, besides proton reduction by hydrogenases.

It is interesting to note that the accumulation of volatile fatty acids (formic, acetic, propionic, butyric) may serve as a substrate for hydrogen production [28], but as an inhibitor to bio-hydrogen production as well [17,18].

There are some drawbacks associated with bio-hydrogen production compared to the traditional methods, i.e., electrolysis. The hydrogenase enzymes are highly sensitive to oxygen. During bio-photolysis, water splits into hydrogen and oxygen. The molar yield of bio-hydrogen cannot exceed 4 moles of hydrogen per mole of glucose along with 2 moles of acetic acid, Equation (1). Next, the hydrogen production rates are less than 1 L/L/h, as shown in [23].

There is a lack of a suitable industrial microbial strains to facilitate the commercialization of bio-hydrogen. One of the well-known and commonly used strains for bio-hydrogen production is Clostridium beijerinckii. According to the literature, the specific molar yield of hydrogen per mole of glucose for this strain is between 1.28 [19] and 2.81 [22]. In other papers, this quantity was 1.97 moles of hydrogen per mole of hexose [24] and 2.52 moles of hydrogen per mole of glucose [25].

The data for bio-hydrogen molar yield with glycerol as the substrate are rather dispersed, from 0.2 to 2.7 moles of hydrogen per mole of glycerol [11–14], with no data available for the strain Clostridium beijerinckii as a strain producer.

There were a series of strains from the genus Clostridium isolated in our institute [29]. They were identified by a molecular–genetic method, described in [30]. The isolated strain Clostridium beijerinckii 6A1 was one of them. It is interesting to estimate its capability to produce hydrogen from cheap and abundant substrates such as glycerol and alcohol stillage in order to utilize these waste streams as a source of energy production.

The aim of the present paper was to test the capability of the new strain Clostridium beijerinckii 6A1 to produce hydrogen from glycerol as a substrate and to compare it to the reference substrate, glucose. Then, the potential of this strain to produce hydrogen from more complex but cheap and abundant substrates such as alcohol stillage was tested too.

The present work studies the production of hydrogen at dark fermentation for three different substrates: glucose, glycerol and stillage remaining after ethanol distillation. The hydrogen potential of the strain Clostridium beijerinckii 6A1 and the possible metabolic routes are tested.

2. Materials and Methods
2.1. Bacterial Strain and Growth Conditions

The bacterial strain was isolated from chickpeas and identified as Clostridium beijerinckii 6A1. The strain was stored at −18 °C in sterile 20% (v/v) glycerol solution in anaerobic environment. Clostridium beijerinckii 6A1 was cultivated under anaerobic conditions at 37 °C, 6.8 pH in RCM medium with the following composition: 5 g L⁻¹ glucose, 10 g L⁻¹ peptone, 13 g L⁻¹ yeast extract, 1 g L⁻¹ soluble starch, 5 g L⁻¹ NaCl, 3 g L⁻¹ sodium acetate, 0.5 g L⁻¹ cysteine hydrochloride.

The isolated strain was identified as Clostridium beijerinckii following the procedure described in [29,30]. The amplified PCR product was sequenced in Macrogen (The Netherlands) using ABI PRISM® 310 DNA Genetic Analyzer, (PE Applied Biosystems, Foster City, CA, USA). The strain was identified as Cl. beijerinckii with 100% similarity.

2.2. Experimental Conditions

A series of experiments were carried out, with three types of substrates as carbon source (alcohol stillage, glucose and glycerol) and correspondingly different ratios and amounts between them. First, pure glucose, 4.5 g/L was used as substrate. Next, mixtures of glucose and alcohol stillage as substrate were tested in the following ratios and amounts:

- glucose 5 g L⁻¹ and 12.5 % (vol.) alcohol stillage; 7.31 g/L total soluble organics;
- glucose 8.5 g L⁻¹ and 25 % (vol.) stillage; 12.62 g/L total soluble organics;
• glucose 6 g L\(^{-1}\) and 25 % alcohol stillage; 10.62 g/L total soluble organics.

The soluble components of the stillage were determined prior to each experiment. For example, they were (in g dm\(^{-3}\)): raffinose, 6.5; lactose, 1.4; glucose, 5.5; acetic acid, 3.8; propionic acid, 0.66; ethanol, 3.6.

Third, pure glycerol at concentrations of 10, 15 and 20 g L\(^{-1}\) was tested as the next substrate. The initial concentrations of the components were estimated after mixing the starting solutions with the inoculum in RCM medium. Temperature of 37 °C was maintained during all runs. The medium acidity of pH 6.8 was adjusted and maintained with phosphate buffer.

All runs lasted 72 h. The hydrogen release took place within the first 24 h. Therefore, the organic loading rates (ORL) for the experiments were considered for the first 24 h. For the three used substrates, OLR (g L\(^{-1}\)d\(^{-1}\)) was estimated as 4.5 for glucose, 7.3, 10.6 and 12.6 for stillage and 10, 15 and 20 g L\(^{-1}\)d\(^{-1}\) for glycerol.

2.3. Hydrogen Production

A sketch of the experimental set-up is shown in Figure 4.

Experiments for bio-production of hydrogen were carried out in a 500 mL dark reactor with a working volume of 400 mL, i.e., with 10 percent free volume. The reactor with the substrate solution within was sterilized prior to the experiments Then, the reactor was purged with nitrogen gas for 15 min for complete oxygen expulsion. After inoculation, the reactor was sealed, wrapped in aluminum foil and immersed in a water bath to maintain a temperature of 37 °C. The resulting amount of hydrogen was passed through 10 M sodium hydroxide solution to capture carbon dioxide. Then, the gas containing hydrogen was collected and measured in a gas holder. Periodically, liquid samples were taken to determine the exhaustion of the used substrates as well as to monitor the metabolites obtained during the fermentation. Then, the gas containing hydrogen was collected and measured in a gas holder. Periodically, liquid samples were taken to determine the exhaustion of the used substrates as well as to monitor the metabolites obtained during the fermentation.
2.4. Analyses

Liquid samples were centrifuged at 11,000 rpm for 6 min and further filtered through a 0.45 µm membrane filter. A high-performance liquid chromatography (HPLC) system (Perkin-Elmer Inc. production, Waltham, MA, USA) with an Aminex Ion Exclusion column HPX-87H (Bio-Rad, Hercules, CA, USA) and an UV-variable wavelength detector (Knauer-wissentschaftliche Geräte GmbH, Berlin, Germany) were used to measure the concentrations of glucose, glycerol and the resulting metabolites (fatty acids and ethanol). A solution of 0.01 N sulfuric acid was used as mobile phase at elution flow rate of 0.6 mL/min. The samples were analyzed in triple, with a scatter ±3%.

3. Results

In all the experiments hydrogen was released within the first 24 h of fermentation. Additional amounts of substrate after the release of hydrogen did not produce further amounts of hydrogen. Therefore, the hydraulic retention time (HRT) was estimated at 24 h.

No pyruvate was observed in the broth in all cases for each substrate. Pyruvate is a key intermediate in all of the mentioned metabolic pathways. One can assume that it is rapidly converted into Acetyl-CoA and then to other intermediates, such as formate, acetate, ethanol, propionate and butyrate. All the processes of hydrogen production, the formation of intermediates and inhibition happened after pyruvate depletion.

3.1. Glucose as Substrate

The experimental results for the intermediate products when glucose was used as the substrate are shown in Figure 5. It is interesting to note that there is a considerable amount of formic acid, whereas the concentrations of acetic and propionic acid are too low. The concentration of ethanol is considerable. The formation of formic acid means that the metabolic pathway for Clostridia shown in Figure 1 is not valid in this case. These observations are in contradiction with the data published for the genus Clostridium claiming that no formic acid is produced when hexoses are used as a substrate [23]. There are indications that bacteria from this genus can produce formic acid [6,9,21,31]. Besides this, more suitable pathways are the ones shown in Figures 2 and 3. However, propionic acid does not appear in the scheme, shown in Figure 3 but it was observed in our experiment, cf. Figure 5. The concentration of formate increased for the first 24 h and remained practically constant for the rest of the experiment.

The review of the obtained results shows that after the direct production of formic acid from glucose, hydrogen is produced via pyruvate only. The amounts of acetic and propionic acids in our case are too low, i.e., 0.012 g dm$^{-3}$ for acetic acid and 0.35 g dm$^{-3}$ for propionic acid. These concentrations were attained after 72 h when the release of hydrogen was completed. No other carboxylic acids were detected, nor acetone, propanol or butanol. No methane was detected in the released gas either.

The results for the hydrogen production rate (in L hydrogen/L medium/h) and the molar hydrogen yield (in mole hydrogen/mole hexose) in our case are 0.052 L/L/h and 2.25 mole hydrogen/mole glucose.

As a reference, the data in the literature for the strain Clostridium beijerinckii show values within 0.19 and 0.35 L/L/h [17] and up to 3.58 mole hydrogen/mole hexose [32].

These results show that most of the hydrogen is produced by the oxidative conversion of pyruvate at the time of hydrogen release. Ethanol is formed by the reductive conversion of Acetyl-CoA, completing the conversion to acetic acid.
3.2. Stillage as Substrate

Some of the stillage components were determined prior to each experiment. For example, they were (in g dm$^{-3}$): raffinose, 6.5; lactose, 1.4; glucose, 5.5; acetic acid, 3.8; propionic acid, 0.66; ethanol, 3.6.

Some results for the intermediate products when stillage was used as substrate are shown in Figure 6.
Here, formic acid is also observed in considerable amounts. It is interesting to note that there are oscillations of glucose and ethanol concentrations. These oscillations can be explained by the input of the hydrolysis of macromolecules of starch hydrolysates as well as of oligosaccharides. They serve as a source of glucose when the present amounts are exhausted because of conversion into intermediates such as ethanol and carboxylic acids. Note that in these cases no other metabolites but formic, acetic and propionic acid and ethanol are produced.

There are similarities in the broth intermediates in the case of glucose as a substrate. There is a difference in the depletion of formate, which was not observed in the case of pH 6.8; RCM medium 280 mL (no glucose). Initial glycerol concentration 20 g/L. Black solid line (●) for hydrogen production. Substrate glycerol, 19.8 g dm⁻³. Some results of experiments when glycerol was used as a substrate are shown in Figure 7. When glycerol was a substrate, the metabolic pathway illustrated in Figure 1 was followed. There was no formic acid, but there were acetic and propionic acid, and some, although small amounts, of butanol noticed at the end, after 72 h. In this case, the concentrations of acetic and propionic acids were considerable, i.e., the pathway inherent to Clostridia shown in Figure 1 is valid.

![Figure 7](image-url)

**Figure 7.** Time profile of variation of the substrate, intermediates and product of dark fermentation for hydrogen production. Substrate glycerol, 19.8 g dm⁻³; 100 mL inoculum in phosphate buffer, pH 6.8; RCM medium 280 mL (no glucose). Initial glycerol concentration 20 g/L. Black solid line (●), glycerol; blue line (●), propionic acid; black solid line (○), ethanol; red solid line (○), acetic acid; black dashed line (○), butanol.
It is interesting that acetate was further degraded whereas ethanol and propionic acid remained constant for the time of experiment. The main reaction of the hydrogen production was the oxidation of $\text{Fd}_{\text{red}}$ by [FeFe]-hydrogenase with the associated consumption of NADH, as stated in articles [6,23,27] and by acetate depletion, cf. Figure 1.

The molar yields for glycerol as a substrate are good for the lowest initial substrate concentration and modest for the higher ones, cf. Table 2. The review of the dependence of the specific molar yield of hydrogen compared to the maximum concentration of acetic and propionic acids shows that the hydrogen yield decreases when the acid concentrations increase. This observation for glycerol as a substrate corresponds to the conclusion of Kim et al. [27] who stated that acetic acid (up to 5000 mg dm$^{-3}$) and butyric acid are inhibitors of hydrogen formation and release. The best results reported for hydrogen molar yields for glycerol as a substrate (i.e., 2.7 and 6 mole H$_2$/mole glycerol) are achieved for other strains, i.e., *Rhodopseudomonas palustris* [14] and *Thermotoga neapolitana* DSM 4359 [33], respectively.

Table 2. Comparison of hydrogen yields from glycerol at different initial substrate concentrations for 24 h.

<table>
<thead>
<tr>
<th>Glycerol Initial Concentration, g dm$^{-3}$</th>
<th>Hydrogen Yield, dm$^{-3}$</th>
<th>Specific Hydrogen Yield, Mole H$_2$/Mole Substrate</th>
<th>Maximum Acetic Acid, g dm$^{-3}$</th>
<th>Maximum Propionic Acid g dm$^{-3}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>9.4</td>
<td>0.08</td>
<td>2.16</td>
<td>0.21</td>
<td>0</td>
</tr>
<tr>
<td>14.4</td>
<td>0.15</td>
<td>0.48</td>
<td>6.84</td>
<td>4.4</td>
</tr>
<tr>
<td>19.8</td>
<td>0.20</td>
<td>0.45</td>
<td>5.40</td>
<td>5.0</td>
</tr>
</tbody>
</table>

The comparison of the hydrogen yields for the studied substrates is shown in Table 3. For stillage as a substrate, the difference between the initial concentration of glucose and the one after 24 h is considered the substrate consumption rate. Comparable results are obtained when glucose or stillage (being starch hydrolysate containing glucose and oligosaccharides) is used as the substrate. In the case of glycerol, higher concentrations of ethanol and propionic acid are observed being competitive to hydrogen production by pyruvate conversion. Moreover, the formation of ethanol and propionic acids are NADH-consuming reactions, thus impeding hydrogen production.

Table 3. Comparison of the hydrogen yields for different substrates for 24 h.

<table>
<thead>
<tr>
<th>Substrate, Initial Concentration, g dm$^{-3}$</th>
<th>Hydrogen Yield, dm$^{-3}$</th>
<th>Specific Hydrogen Molar Yield, Mole H$_2$/Mole Substrate</th>
<th>Maximum Acetic Acid, g dm$^{-3}$</th>
<th>Maximum Propionic Acid g dm$^{-3}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose, 4.5</td>
<td>0.5</td>
<td>2.23</td>
<td>0.12</td>
<td>0</td>
</tr>
<tr>
<td>Glucose, stillage, 4.6</td>
<td>0.0245</td>
<td>2.03</td>
<td>0.56</td>
<td>0</td>
</tr>
<tr>
<td>Glucose, stillage, 5</td>
<td>0.25</td>
<td>2.0</td>
<td>0.61</td>
<td>0</td>
</tr>
<tr>
<td>Glycerol, 9.4</td>
<td>0.08</td>
<td>2.16</td>
<td>0.21</td>
<td>0</td>
</tr>
<tr>
<td>Glycerol, 14.4</td>
<td>0.15</td>
<td>0.47</td>
<td>6.84</td>
<td>2.8</td>
</tr>
<tr>
<td>Glycerol, 19.8</td>
<td>0.20</td>
<td>0.43</td>
<td>5.4</td>
<td>5.0</td>
</tr>
</tbody>
</table>

The values obtained for the specific hydrogen molar yield were processed by the Student test. It was established that with a probability of 95% the mean of the specific molar hydrogen yield (in mole H$_2$/mole substrate) for all substrates was a 2.11 ± 0.11 confidence interval. For the case of higher concentrations of glycerol, this value was 0.46 ± 0.19. Hence, there is no overlapping of the confidence intervals for these two groups of processes and they should be considered separately.

The comparison of the maximum molar hydrogen yields with those shown in the literature for the same strain shows a reasonable fit, cf. Table 4. For example, the molar
yield of hydrogen per mole hexose for this strain is between 1.16 and 3.58 mole H₂/mole glucose when a culture of Clostridium beijerinckii has been used. The best result is 3.58 mole H₂/mole glucose reported in [32].

Table 4. Comparison of best of the obtained data for the specific hydrogen yield and the hydrogen production rate with data available in the literature for dark fermentation by the strain Clostridium beijerinckii.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Specific Hydrogen Yield, Mole H₂/Mole Substrate</th>
<th>Hydrogen Production Rate L/L Medium/h</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>3.58</td>
<td>0.35</td>
<td>[32]</td>
</tr>
<tr>
<td>Glucose</td>
<td>2.81</td>
<td>-</td>
<td>[22]</td>
</tr>
<tr>
<td>Glucose</td>
<td>2.52</td>
<td>0.39</td>
<td>[25]</td>
</tr>
<tr>
<td>Glucose</td>
<td>2.23</td>
<td>0.052</td>
<td>This work</td>
</tr>
<tr>
<td>Glucose</td>
<td>2.0</td>
<td>0.66</td>
<td>[21]</td>
</tr>
<tr>
<td>Glucose</td>
<td>1.97</td>
<td>-</td>
<td>[24]</td>
</tr>
<tr>
<td>Glucose</td>
<td>1.16</td>
<td>0.19</td>
<td>[34]</td>
</tr>
<tr>
<td>Alcohol stillage</td>
<td>2.03</td>
<td>0.025</td>
<td>This work</td>
</tr>
<tr>
<td>Starch</td>
<td>1.8</td>
<td>0.41</td>
<td>[21]</td>
</tr>
<tr>
<td>Food waste</td>
<td>-</td>
<td>0.108</td>
<td>[27]</td>
</tr>
<tr>
<td>Glycerol</td>
<td>2.73</td>
<td>-</td>
<td>[13]</td>
</tr>
<tr>
<td>Glycerol</td>
<td>2.16</td>
<td>0.009</td>
<td>This work</td>
</tr>
<tr>
<td>Glycerol</td>
<td>1.21</td>
<td>-</td>
<td>[35]</td>
</tr>
</tbody>
</table>

However, the hydrogen production rates obtained in the present work for glycerol and stillage as substrates are considerably lower than those reported in the literature.

Our results show that the biological processes for hydrogen production are superior to the traditional ones for a point of view energy input. The hydrogen bio-production rates and the process efficiency must be enhanced to meet the practical demands of produced hydrogen.

One must bear in mind that the energy efficiency for dark fermentation is about 9.6% and for a two-stage one it is 27.2% [7]. The energy efficiency for steam methane reforming reaches 64%, but with high greenhouse gas emissions.

Our efforts in future should be directed toward increasing the hydrogen production rate through the optimization of the fermentation process by adopting two-stage dark- and photo-fermentation or by selection of a better hydrogen-producing strain.

4. Conclusions

A newly isolated strain, Clostridium beijerinckii 6A1, was tested under batch conditions for hydrogen production capacity with glucose, glycerol and alcohol stillage as substrates.

Relatively high molar yields of hydrogen (up to 2.23 mole H₂/mole glucose) and modest hydrogen production rates (0.052 L hydrogen/L medium/h) were achieved when glucose was used as a substrate. Similar results for the specific molar yield of hydrogen were achieved when stillage and glycerol (at low initial concentration) were used as substrates. The hydrogen production rate was much lower than the reported ones in the literature.

At higher initial concentrations of glycerol, the hydrogen yields were considerably lower because of accumulated acetate and propionate, being inhibitors to hydrogen production and because of the excessive consumption of NADH.

In all cases, the evolution of hydrogen takes place during the first 24 h.

It is evident in our experiments that there is a neat difference in the metabolic pathways of substrate digestion when hexose-based substrate or glycerol are used. The products
of glycerol digestion show that the pathway with no formic acid is observed and small amounts of butanol are detected.

When glucose or hexose-based substrates such alcohol stillage were used, formic acid formation was observed, i.e., the pathway involving fatty acids and ethanol formation was appropriate for the observed process of hydrogen release.

In all cases, substrate digestion stopped the accumulation of acetic acid, propionic acid and ethanol. Probably, these intermediates are inhibitors to the further digestion to other products.

Efforts must be concentrated toward the enhancement of the hydrogen production rate and the process efficiency for the practical application of the studied strain in the future.

Author Contributions: Conceptualization, V.B.; methodology, V.B.; investigation, E.V. and T.P.-M.; data curation, E.V. and T.P.-M.; writing—original draft preparation, V.B.; writing and editing, V.B.; project administration, V.B.; funding acquisition, V.B. All authors have read and agreed to the published version of the manuscript.

Funding: The authors kindly acknowledge the financial support of project № BG05M2OP001-1.002-0014 “Center of competence HITMOBIL—Technologies and systems for generation, storage and consumption of clean energy”, funded by Operational Programme “Science and Education For Smart Growth” 2014–2020, co-funded by the EU from European Regional Development Fund.

Data Availability Statement: Not applicable.

Acknowledgments: The authors appreciate the support of D. Yankov and his group for supplying us with the strain Clostridium beijerinckii 6A1.

Conflicts of Interest: The authors declare no conflict of interest.

References


26. Gomez-Flores, M.; Nakhta, G.; Hafez, H. Hydrogen production and microbial kinetics of Clostridium termitidis in mono-culture and co-culture with Clostridium beijerinckii on cellulose. AMB Express 2017, 7, 84. [CrossRef]


35. Tchounian, K.; Muller, N.; Schink, B.; Tchounian, A. Glycerol and mixture of carbon sources conversion to hydrogen by Clostridium beijerinckii DSM791 and effects of various heavy metals on hydrogenase activity. Int. J. Hydrogen Energy 2017, 42, 7875–7882. [CrossRef]

Disclaimer/Publisher's Note: The statements, opinions and data contained in all publications are solely those of the individual author(s) and contributor(s) and not of MDPI and/or the editor(s). MDPI and/or the editor(s) disclaim responsibility for any injury to people or property resulting from any ideas, methods, instructions or products referred to in the content.